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Crystallization and initial crystal characterization of the C-terminal phosphoglycerate mutase homology domain of Sts-1

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Sts-1 is a multidomain protein that plays an important role in T-cell signaling. Sts-1 contains a ubiquitin-association (UBA) domain at the N-terminus, followed by an Src homology-3 (SH3) domain and a C-terminal domain that shares sequence homology to phosphoglycerate mutases (PGMs). The C-terminal domain of Sts-1, Sts-1_{PGM}, crystallizes in space group $C2$ with two different crystal forms. The first crystal form contains two or three Sts-1_{PGM} molecules in the asymmetric unit and diffracts to 1.82 Å resolution, with unit-cell parameters $a = 116.2$, $b = 74.3$, $c = 100.1$ Å, $\alpha = \gamma = 90$, $\beta = 101.5^\circ$. The second crystal form contains four or six Sts-1_{PGM} molecules in the asymmetric unit, with unit-cell parameters $a = 214.9$, $b = 75.1$, $c = 116.4$ Å, $\alpha = \gamma = 90$, $\beta = 111.6^\circ$. Greater than 95% complete native and SeMet data sets have been collected and structure determination using the multiple anomalous dispersion (MAD) technique is ongoing.

1. Introduction

Within the mammalian immune system, T cells play a central role in the recognition and elimination of invasive micro-organisms. However, they also pose a potential danger to their host in that many of the properties that make them effective mediators of the immune response can be turned inadvertently against host tissue (reviewed in Walker & Abbas, 2002; Ohashi, 2002). Not surprisingly, the immune system has evolved a variety of regulatory mechanisms to ensure that T cells target foreign antigens with remarkable fidelity and selectivity (Sebzda *et al.*, 1999; Singer & Koretzky, 2002; Schwartz, 2005). When any of these mechanisms breaks down, varied pathologies ranging from immunodeficiencies to autoimmune disorders are known to develop (Rose, 2002).

To detect foreign pathogens within the body, T cells utilize a receptor known as the T-cell receptor or TCR. Engagement of the TCR activates numerous intracellular signaling cascades (Germain & Stefanova, 1999). Tyrosine kinases such as Lck and Zap-70 play a critical role in propagating downstream signals (Latour & Veillette, 2001). A variety of adaptor and scaffolding proteins also participate in TCR signaling (Samelson, 2002; Koretzky & Myung, 2001). Additional signaling elements include lipid kinases, Tec-family kinases and components of both the Ca^{2+}-calcineurin and MAPK signaling cascades (Cantrell, 1996). To limit the intensity of the signals generated by receptor activation, T cells possess a number of negative regulatory mechanisms (Veillette *et al.*, 2002). For example, the tyrosine kinases involved in TCR-mediated signaling are subject to several levels of regulation. These include dephosphorylation of activating residues by tyrosine phosphatases such as Shp-1 and phosphorylation of inhibitory residues by tyrosine kinases such as Csk. Another class of enzymes, the ubiquitin ligases, negatively regulates TCR signaling by targeting specific proteins for degradation (Duan *et al.*, 2004). Recently, proteins belonging to the suppressor of TCR signaling (Sts) family were discovered and their role in negatively regulating TCR signaling pathways was identified (Carpino *et al.*, 2004). However, the mechanism(s) of action of the Sts proteins remains to be elucidated.

Members of the *Sts* gene family can be found in an evolutionarily diverse group of organisms. Most family members are characterized by a unique tripartite structure, with an N-terminal UBA (ubiquitin-

association) domain, a central SH3 (Src-homology 3) domain and a carboxyl region with similarity to the catalytic domain of members of the bis-phosphoglycerate mutase (PGM) family (Kowanetz *et al.*, 2004; Feshchenko *et al.*, 2004; Fig. 1). The PGM motif is found in a group of structurally related enzymes that catalyze the transfer of phosphate from small molecules such as phosphoglycerate and fructose-2,6-phosphate (Jedrzejas, 2000). The realization that Sts proteins play a role in regulating TCR signaling pathways emerged from an analysis of mice lacking both Sts-1 and Sts-2. Specifically, naïve peripheral T cells isolated from Sts-1/2$^{-/-}$ mice display a striking hypersensitivity to T-cell receptor stimulation. This hypersensitivity is manifested by pronounced increases in TCR-induced proliferation and cytokine production by mutant T cells relative to wild-type T cells. In addition, mutant mice demonstrate increased susceptibility to autoimmunity in a mouse model of multiple sclerosis (Carpino *et al.*, 2004). These results argue that Sts-1 and Sts-2 are critical negative regulators of the signaling pathways that participate in the activation of naïve peripheral T cells.

Recently, our efforts have focused on elucidating the mechanism of action of the Sts proteins. Towards this end, we initiated a crystallographic study to determine the three-dimensional structure of Sts-1. Here, we present a preliminary crystallographic characterization of the C-terminal phosphoglycerate mutase domain of mouse Sts-1, Sts-1$_{PGM}$.

2. Materials and methods

2.1. Expression and purification

The Sts-1$_{PGM}$ domain (residues 369–640) was cloned as an N-terminally His-tagged protein in the pProEX-HTb vector (Life Technologies) and expressed in *Escherichia coli* BL21-CodonPlus strain (Stratagene). The engineered protein contained the sequence HHHHHHDYIPTTENLYFQGAMGS N-terminal to Gly369. Bacterial cultures were grown in Luria–Bertani broth supplemented with 50 mg l^{-1} ampicillin to an optical density at 600 nm of 0.5–0.7, at which point protein expression was induced with 75 mg l^{-1} isopropyl thiogalactopyranoside (IPTG) at 291 K overnight. The cultures were pelleted and cells were washed with lysis buffer (20 m*M* Tris–HCl, 150 m*M* NaCl, 5 m*M* imidazole pH 7.5, 0.1 m*M* phenylmethylsulfonyl fluoride) and stored at 193 K. For purification, freshly thawed cells were suspended in lysis buffer and ruptured in a French press. Clarified cell lysates were passed over an Ni–NTA matrix (Qiagen) equilibrated with buffer *A* (20 m*M* Tris–HCl, 500 m*M* NaCl pH 8.0, 5 m*M* imidazole). Non-specifically bound proteins were eluted with buffer *A* supplemented with 20 m*M* imidazole. His$_6$-Sts-1$_{PGM}$ was eluted off the column by stepping the imidazole concentration to 250 m*M* in buffer *A*. His$_6$-Sts-1$_{PGM}$ was dialyzed overnight at 277 K in a buffer consisting of 20 m*M* Tris–HCl pH 8.0, 300 m*M* NaCl, 0.1 m*M* EDTA and 1 m*M* DTT. During dialysis, recombinant His$_6$-tagged TEV protease (Nallamsetty *et al.*, 2004) was added to the His$_6$-Sts-1$_{PGM}$ at a molar ratio of 1:20 (TEV:His$_6$-Sts-1$_{PGM}$) in order to remove the His$_6$ tag from Sts-1$_{PGM}$. The cut protein did not contain any extra amino acids N-terminal of Gly369. Sts-1$_{PGM}$ was repassed over an Ni–NTA column to remove uncut proteins and His$_6$-TEV protease. Untagged Sts-1$_{PGM}$ was concentrated to ∼20 mg ml^{-1} and further purified over a Superdex 200 (GE Healthcare) size-exclusion column equilibrated with 10 m*M* HEPES pH 7.5, 150 m*M* NaCl, 5 m*M* β-mercaptoethanol. Judging from the size-exclusion column elution profile, Sts-1$_{PGM}$ eluted as an ∼60 kDa protein consistent with a dimer. Sts-1$_{PGM}$ was concentrated to ∼20 mg ml^{-1} by centrifugation using Amicon Centriprep concentrators (10 kDa molecular-weight cutoff), flash-frozen in liquid nitrogen and stored at 193 K.

SeMet-Sts-1$_{PGM}$ protein was generated by inhibiting the methionine-synthesis pathway during bacterial growth and substituting SeMet for Met (Doublié, 1997). Cells were grown in minimal medium supplemented with 60 mg l^{-1} seleno-L-methionine (Sigma). Initial cultures (0.1 l) were grown in Luria–Bertani broth. Before induction of expression, the cells were centrifuged for 15 min at 2700*g*, washed twice with 25 ml minimal medium and resuspended in SeMet-containing minimal medium. Growth conditions and conditions for induction of expression were as described for the wild-type native protein. SeMet-enriched Sts-1$_{PGM}$ was purified as described for the wild-type native protein.

Purified Sts-1$_{PGM}$ was better than 95% pure as assessed by SDS–PAGE. The molecular weights of normal wild-type (30 864 Da) and SeMet-enriched (31 067 Da) Sts-1$_{PGM}$ were determined by electrospray mass spectrometry (Proteomics Centre, Stony Brook University). The mass spectrum of the SeMet protein confirmed that the four Met residues have been fully substituted by SeMet.

2.2. Crystallization and data collection

Crystallization screens of Sts-1$_{PGM}$ were performed at 293 K using the hanging-drop vapor-diffusion method. Crystals were obtained within several hours when Sts-1$_{PGM}$ or SeMet-Sts-1$_{PGM}$ (10 mg ml^{-1}) was mixed with equal amounts of a solution consisting of 11–14% polyethylene glycol (PEG 8000, Fluka), 0.2 or 0.3 *M* $MgCl_2$ or magnesium acetate, 0.1 *M* HEPES pH 7.0, 5%(*w*/*v*) ethylene glycol and equilibrated against 1 ml reservoirs of the same solution.

Figure 1
Alignment between PGM-like domains of mSts-1 and the *E. coli* cofactor-dependent phosphoglycerate mutase (PDB code 1e58, chain *A*). The two sequences were aligned using the *T-Coffee* program (Notredame *et al.*, 2000; http://ch.embnet.org/). Shaded residues denote homologous regions, with the intensity of the shading correlating with the relative degree of homology. The sequence 'RGHE' is the phosphoglycerate mutase family signature.

Table 1
Statistics of data collection and processing.

Values for the highest resolution shell are given in parentheses. The highest resolution shells are 2.07–2.0 Å for the peak and the inflection wavelengths and 2.28–2.20 Å for the remote data.

		SeMet		
	Native	Peak	Inflection	Remote
NSLS beamline	X26C	X25	X25	X25
Space group	*C*2	*C*2	*C*2	*C*2
Wavelength (Å)	1.0	0.9799	0.9801	0.95
Unit-cell parameters				
a (Å)	116.2	214.9	215.0	215.2
b (Å)	74.3	75.1	75.2	75.2
c (Å)	100.1	116.4	116.4	116.4
β (°)	100.5	111.6	111.6	111.6
Resolution range (Å)	50–1.82	50–2.0	50–2.0	50–2.2
Observations	770707	2357491	2364548	2053208
Unique reflections	72288	114466	115373	86664
$\langle I\rangle/\langle\sigma(I)\rangle$	26.0 (2.6)	29.6 (3.4)	29.8 (3.2)	33.5 (4.5)
Completeness (%)	96.3 (94.4)	99.3 (98.3)	99.1 (97.9)	99.3 (98.1)
$R_{\rm merge}$ (%)	5.7 (57.2)	8.1 (59.6)	7.9 (68.0)	7.8 (57.7)

† $R_{\rm merge}(I) = \sum_h \sum_i |I_i(h) - \langle I(h)\rangle| / \sum_h \sum_i I_i(h)$, where $\langle I(h)\rangle$ is the mean intensity for the set of equivalent reflections defined by the *C*2 space group for the reflections denoted by $I_i(h)$.

Wild-type or SeMet-enriched Sts-$1_{\rm PGM}$ crystals were transferred into a solution of 15% PEG 8000, 0.2 or 0.3 *M* $MgCl_2$ or magnesium acetate, 0.1 *M* HEPES pH 7.0 and 5% ethylene glycol. Cryoprotection was achieved by increasing the PEG concentration to 30% in steps of 5% and equilibrating the crystal for a few minutes at each step. Crystals were mounted on a nylon loop (Hampton Research Inc.) before freezing in liquid nitrogen. Native and MAD data sets to 1.82 and 2.0 Å resolution were collected on a 2k × 2k or 3k × 3k ADSC CCD detector at beamline X26C or X25 at the National Synchrotron Light Source (NSLS; Brookhaven National Laboratory). All intensities were indexed, processed and scaled with the *HKL*2000 package (Otwinowski & Minor, 1997).

3. Results and discussion

Purification of the C-terminal phosphoglycerate mutase domain of mouse Sts-1 (Sts-$1_{\rm PGM}$) wild-type or SeMet-substituted protein yielded approximately 50 mg of pure protein per litre of bacterial cell culture. The size-exclusion profile is compatible with Sts-$1_{\rm PGM}$ being a dimer. Sts-$1_{\rm PGM}$ crystals appeared when a solution of 11–14% PEG 8000, 0.1 *M* HEPES pH 7.0 with various salts (sodium acetate, ammonium sulfate *etc.*) was used as precipitant. Since well shaped monocrystals appeared in the presence of $MgCl_2$ or magnesium acetate (Fig. 2), these two salts were used subsequently to crystallize the wild-type native or SeMet-substituted protein. Sts-$1_{\rm PGM}$ crystallized in two crystal forms that belonged to the monoclinic space group *C*2. Form I is characterized by unit-cell parameters $a = 116.2$, $b = 74.3$, $c = 100.1$ Å, $\alpha = \gamma = 90$, $\beta = 101.5^\circ$, which are compatible with the presence of either one or one and a half Sts-$1_{\rm PGM}$ dimers in the asymmetric unit, corresponding to a Matthews coefficient $V_{\rm M}$ of 3.43 or 2.41 Å^3 Da^{-1} and a solvent content of 64 or 46%, respectively. Form II is characterized by unit-cell parameters $a = 214.9$, $b = 75.1$, $c = 116.4$ Å, $\alpha = \gamma = 90$, $\beta = 111.6^\circ$, which are compatible with the presence of two or three dimers in the asymmetric unit, corresponding to $V_{\rm M}$ values of 3.54 or 2.36 Å^3 Da^{-1} and solvent contents of 65 or 48%, respectively. Both crystal forms have the same shape and there is no strong evidence to suggest that they coexist in a given drop. However, we noticed that when a 0.3 *M* salt concentration was used in the crystallization and in the cryoprotection solutions, regardless of the nature of the magnesium salt, the crystals were consistently more stable and more likely to have the large unit cell characteristic of form II.

Figure 2
Typical crystals (0.1 × 0.1 × 0.3 mm) of Sts-$1_{\rm PGM}$ obtained by the hanging-drop method.

Data extending to 1.82 Å resolution were collected at 100 K from one form I crystal at beamline X26C (NSLS) and were processed with *HKL*2000. Diffraction data to 2.0 Å resolution were collected at beamline X25 (NSLS) on an SeMet-substituted crystal at wavelengths of 0.9799, 0.9801 and 0.95 Å, which correspond to the peak, inflection and remote wavelengths of the selenium edge. Statistics of data collection and processing are shown in Table 1. Structure solution of Sts-$1_{\rm PGM}$ is in progress using the multiple anomalous diffusion (MAD) technique.

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